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CROP SCIENCE

Stigma structure and receptivity in papaya (*Carica papaya* L.)

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Abstract: The objective of this study was to evaluate the stigma morphoanatomy and receptivity in ten promising papaya accessions, to expand knowledge useful for genetic improvement of the culture. The morphoanatomy was analyzed by light and scanning electron microscopy, and the stigma receptivity was investigated by application of hydrogen peroxide and α-naphthyl-acetate with fast blue B salt, in pre-anthesis, anthesis and post-anthesis. The papaya accessions presented dry stigma surface, presence of one to six erect stigmatic lobes, with structures joined to the upper base of the style and covered with numerous elongated unicellular tubular papillae, distributed on both faces of the epidermis. The morphoanatomy had a similar pattern in all the accessions, differing only in the timing of floral development. The stigma receptivity in some accessions occurred even before floral opening, continuing with greater intensity in anthesis and post-anthesis. Pre-anthesis is the stage least propitious for controlled hybridizations due to the weak or absent stigmatic receptivity. The results obtained provide information on the floral stage that is most propitious for fertilization, as well as supporting future investigations of the botanical morphology of the species.

Key words: Caricaceae, floral anthesis, histology, hydrogen peroxide, α-naphthyl-acetate, scanning electron microscopy.

INTRODUCTION

Carica papaya L. is the only species of Caricaceae Dumort. that produces marketable fruit. Since 2015, Brazil has been the world's second largest producer of papaya, accounting for 8.12% of global output, corresponding to 13.02 million metric tons (Faostat 2019). The fruits with greatest acceptance in the Brazilian market come from hermaphrodite plants, basically due to the type of flowers, which favor specific traits of the fruit, such as: oblong to pyriform shape; small internal cavity; and high pulp thickness (Costa & Pacova 2003, Ming et al. 2007).

At present, programs for genetic improvement of papaya are focused on obtaining new varieties that are resistant both to biotic and abiotic factors and that meet the requirements of the domestic and external markets as well as of farmers (Dantas & Lima 2001, Ruggiero et al. 2011). Understanding the reproductive biological aspects of different accessions of a species helps to identify potential parents for use in future crosses and/or hybridizations (Bernardello et al. 2001, Souza et al. 2017). Knowledge of the morphoanatomy and stigma receptivity of different accessions of *C. papaya* has been important to support actions involving controlled pollinations. Besides this, studies of these aspects improve understanding of the compatibility between pollen grains and stigmas, thus influencing the fertilization rate and formation of fruits and seeds (Galen et al. 1987, Lenzi & Orth 2004).

Stigma receptivity is a process that can last several days, or only a few hours (Heslop-Harrison 1992), since it is associated with the activities of various enzymes, such as peroxidase, esterase and dehydrogenase. These enzymes can be produced in the different stages of floral development (Heslop-Harrison & Shivanna 1977, Knox 1984, Galen & Plowright 1987, Shivana & Rangaswamy 1992, Dafni & Maués 1998). The corresponding enzymatic processes are responsible for factors that influence the success of germination of the pollen grains, development/penetration of the pollen tube in the stigma, and probably the incompatibility responses between possible parents of interest (Heslop-Harrison et al. 1975, Kulloli et al. 2010, Souza et al. 2017).

Stigma receptivity can be measured in various ways. The use of hydrogen peroxide (H_2O_2) is one of the most widespread techniques, due to its easy performance and good applicability, besides providing an immediate result at low cost. This solution contains hydrogen peroxide that when in contact with peroxidases present on the stigmas creates a reaction when it is receptive promoting the formation of air bubbles that can be easily observed by the naked eye (Kearns & Inouye 1993). Another method to assess stigma receptivity is the reaction of benzidine + hydrogen peroxide (Dafni & Maués 1998), which causes receptive stigmas to change from their natural color to dark blue, besides promoting the formation of easily observed air bubbles due to the action of the peroxidases contained in the hydrogen peroxide. However, the method relying on the reaction of α-naphthyl-acetate with fast blue B salt is considered more reliable (albeit more expensive), because the action of this solution is based on the esterase reaction, avoiding false-positive results, by changing the stigma's natural color to a darker shade or black when receptive (Kearns & Inouye 1993, Souza et al. 2016, Soares et al. 2018).

Since there are no reports (to the best of our knowledge) of this type of study involving papaya germplasm, the objective of this work was to evaluate the morphoanatomy and stigma receptivity in different papaya accessions (*C. papaya*) belonging to the Active Germplasm Bank of the Embrapa Cassava and Fruits research unit (*Embrapa Mandioca e Fruticultura*) to expand knowledge to enable actions for genetic improvement.

MATERIALS AND METHODS

We evaluated 10 papaya accessions of the species *Carica papaya* L., four of the 'Solo' type (CMF-020, CMF-026, CMF-070 and CMF-123) and six of the 'Formosa' type (CMF-022, CMF-055, CMF-075, CMF-142, CMF-245 and CMF-247), belonging to the Papaya Active Germplasm Bank (Papaya AGB) of Embrapa Mandioca e Fruticultura, located in the municipality of Cruz das Almas, Bahia, Brazil. The accessions were selected based on studies aiming at subsequent establishment of a nuclear collection, i.e., a limited group of accessions that represent the maximum conserved genetic diversity of a species. The collection was carried out in January 2017 and the stigmas were removed from hermaphrodite flowers.

Morphoanatomy of the stigma

For morphological characterization, the stigmas from hermaphrodite flowers in floral anthesis were collected from each accession and immediately fixed in modified Karnovsky's solution (Karnovsky 1965) (2% glutaraldehyde, 2% paraformaldehyde, $Cacl_2$ 0.001 M and sodium cacodylate buffer 0.05 M) with pH adjusted to 7.2, for a period of 48 hours. Then the stigmas were

dehydrated in an increasing ethyl series (35-100%), for 20 minutes each. The samples were dried to critical point, placed on metal supports and sputter coated with gold for analysis under an LEO 435 VP (variable pressure) scanning electron microscope (Carl Zeiss, Jena, Germany).

For anatomical characterization, the stigmas were also fixed in Karnovsky's solution and dehydrated in an increasing ethyl series (35-100%). Then they were infiltrated and embedded in resin using the HistoResin kit (Leica, Heidelberg, Germany).

The resin was polymerized at room temperature for 48 hours and the samples were then placed on wood supports and serial histological sections (4-5 µm) were obtained with a Leica RM 2155 rotary microtome (Leica, Nussloch, Germany). The best sections were placed on slides and stained with acid fuchsin (0.1% p/v) followed by toluidine blue (0.05% p/v) (Feder & O'Brien 1968). The prepared sections were analyzed and photographed with an Axiosckop 2 photomicroscope (Carl Zeiss, Germany).

The length, width and diameter of the stigma and style were measured, expressed in millimeters, based on SEM images with the aid of the ImageJ 1.46r software (Rasband 2012). The data were submitted to analysis of variance and the means were compared by the Scott-Knott test ($p<0.01$). The statistical analyses were performed with the R program (R Core Team 2017).

Stigma receptivity

The stigma receptivity was evaluated at three floral development stages: pre-anthesis (5:00 p.m. of the day before), anthesis (7:30 a.m.) and post-anthesis (24 hours after anthesis), with three repetitions, using two methods.

Method 1 consisted of immersing the stigmas in α-naphthyl-acetate with phosphate buffer, acetone and fast blue B salt for 5 minutes. The receptivity was assessed by visual observation of a change from the natural surface color to darker shades with different scales (Pearse 1972, Dafni 1992).

Method 2 consisted of immersion of the stigmas in hydrogen peroxide (H_2O_2) for approximately 3 minutes and observation of the presence or absence of air bubbles with the naked eye. This reaction occurs due to the contact of the hydrogen peroxide with the peroxidases present in the receptive stigma (Zeisler 1938). When using this method, care must be taken when removing the flower petals so that no cuts or other damages are caused to the stigmas, to avoid false-positive results.

The stigma receptivity was evaluated by the method adapted by Dafni & Maués (1998), whereby scores are assigned (0, 1, 2 or 3). The average of the repetitions resulted in a classification of the intensity of receptivity based on the scale indicated in Table I.

The experimental design was completely randomized in a 3 x 10 factorial scheme (floral development stages x accessions), with five repetitions for each treatment, where each repetition consisted of one flower. As before, the data were submitted to analysis of variance and the means were compared by the Scott-Knott test ($p<0.01$). The statistical analyses were performed with the R program (R Core Team 2017).

RESULTS AND DISCUSSION

Morphoanatomy of the stigma

The morphoanatomical observations revealed that the stigma surfaces of all the *C. papaya* accessions were dry, with the presence of six well-developed erect stigmatic lobes, with structures joined to the upper base of the style and covered with numerous papillae (Figures 1-2). The observation of dry stigmas with

Table I. Scores to define the intensity of the stigma receptivity in papaya flowers.

Scores	Scale of scores	Intensity of receptivity
0	0 – 0.33	No reaction
1	0.34 – 1.33	Weak positive reaction (+)
2	1.34 – 2.66	Strong positive reaction (++)
3	2.67 – 3.00	Very strong positive reaction (+++)

unicellular papillae of *Carica* species was also described by Heslop-Harrison & Shivanna (1977) and Damasceno Junior et al. (2009). The latter authors stated that the hydration and interaction of the pollen grains on the stigma occur due to the presence of a large amount of pollenkitt on the grains' surfaces, facilitating adherence on the style. The stickiness of this substance favors adherence of the pollen grains to the bodies of pollinators. It also protects against dehydration of the pollen grains and thus prevents loss of viability, and promotes recognition of the pollen by the stigma (Lin et al. 2013), maximizing pollen germination and fertilization of the ovule (Lam et al. 2005).

The examination of the sections by light microscopy and of the samples by scanning electron microscopy revealed the presence of slightly twisted and amply flattened stigmatic lobes and invaginations ranging from one to six, unequal in the same flower, with the appearance of moose antlers. This trait means the stigma has larger surface area for contact and adherence of the pollen grains.

The morphometric analysis allowed quantitatively characterizing the stigmas and styles (Table II). There were significant differences between the plants of the 'Solo' and 'Formosa' groups for the numbers of stigmatic lobes and styles, while no significant differences were observed for the number of stigmatic papillae.

In general, the accessions of the 'Formosa' group had larger stigmas and styles than those of the 'Solo' group. These differences in size, almost imperceptible to the naked eye (2 mm), were evident in the SEM images and can possibly be related to the overall size of the flower and the fruits generated by each group. The 'Solo' group is represented by the 'Golden' and 'Sunrise Solo' lineages, which generally produce small fruits with weights varying between 300 and 650 g. In turn, the "Formosa' group, composed by the hybrids 'Tainung 1' and 'Calimosa', produces larger fruits, weighing between 1.0 and 1.3 kg (Dias et al. 2011).

Another morphological trait observed on the surface of the stigmas was the presence of papillae on the adaxial face, lateral face and a portion of the abaxial face (Figures 1-2). These papillae were unicellular and had an oblong tubular shape with swollen apex (Figures 1-2). The length and diameter of the papillae varied according to the accessions, with average lengths ranging from 125.73 µm (CMF-245) to 146.72 µm (CMF-075) and diameters from 20.73 µm (CMF-247) to 32.27 µm (CMF-075). These trichomes were prominent on the walls of the epidermal cells.

Beneath the stigmatic papillae and epidermis, the parenchyma presented small intercellular voids distributed irregularly with numerous vascular bundles arranged in two rows per stigmatic lobe (Figures 1-2).

In all the *C. papaya* accessions, the stigma and style were formed of a unistratified epidermis covered by a thin striated cuticle (Figures 1-2). The internal epidermal cells had dense cytoplasm, prominent nucleus and smooth cuticle. The styles in general were short,

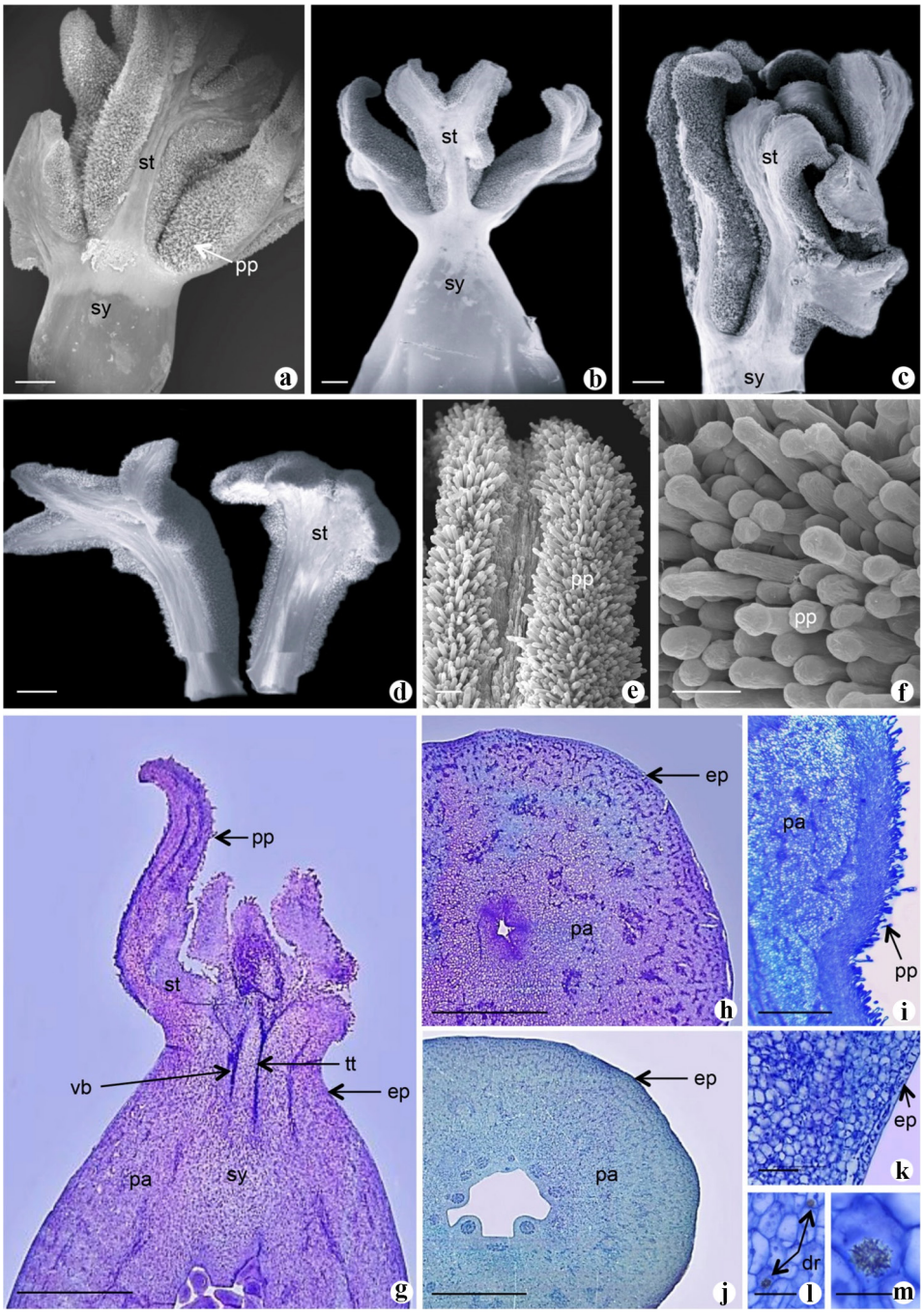

Figure 1. Morphoanatomy of the stigma/style in different papaya accessions (*C. papaya* L.) of the 'Solo' group. a) CMF-020; b) CMF-026; c) CMF-070; d-f) CMF-123; g) CMF-020; h) CMF-026; i) CMF-070; j) CMF-123; k-m) CMF-020. a-f) Morphological traits observed by scanning electron microscopy (SEM). g-m) Anatomic sections observed by light microscopy (LM). d) Detail of the stigmatic lobes. e-f) Stigmatic papillae. g) Longitudinal anatomic sections showing the transmitting tissue and stigmatic papillae. h) Transversal anatomic section of the middle region of the style. i) Anatomic section showing the stigmatic papillae. j) Transversal anatomic section of the base of the style. k) Anatomic section showing the abaxial epidermis of the stigmatic lobes. l-m) Idioblasts containing druses in the stigmatic lobes. ep = epidermis, pa = parenchyma, sp = stigmatic papillae st = stigma, sty = style, tt = transmitting tissue, vb = vascular bundle, dr = druses. Bars: a-d) = 500 µm; e) = 100 µm; f, i, k, l) = 50 µm; g-h, j) = 200 µm; m) = 20 µm.

Table II. Morphometry of the stigma and style in 10 *Carica papaya* accessions from the Active Germplasm Bank of Embrapa Mandioca e Fruticultura.

Accessions	Stigmatic lobes[1] (µm)			Stigmatic papillae (µm)		Style (µm)	
	Length**	Diam.**	Width**	Length[ns]	Diam.[ns]	Length**	Diam.**
'Solo' Group							
CMF-020	4,183.92b	3,284.83b	601.36b	131.84	25.63	2,332.48b	2,321.34b
CMF-026	3,836.39b	2,223.51b	523.12b	128.85	23.23	1,161.79b	1,672.79b
CMF-070	4,212.23b	3,221.56b	591.32b	142.12	28.12	2,221.45b	2,152.18b
CMF-123	4,123.56b	3,128.64b	589.63b	136.82	27.73	2,432.34b	2,248.28b
'Formosa' Group							
CMF-022	6,153.67a	3,982.56a	732.72a	128.63	21.27	2,945.73a	2,523.94a
CMF-055	5,923.83a	4,193.74a	652.64a	138.64	29.72	3,738.82a	3,365.93a
CMF-075	6,328.83a	4,725.45a	748.34a	146.72	32.27	2,995.72a	3,637.82a
CMF-142	5,437.25a	3,982.47a	632.74a	128.93	24.36	3,582.42a	2,983.48a
CMF-245	5,227.63a	3,862.43a	587.73b	125.73	23.67	3,297.74a	2,678.86a
CMF-247	6,029.73a	4,298.63a	683.74a	147.79	20.73	3,763.87a	3,245.74a

**** Means followed by the same letter in the column do not differ by the Scott-Knott test (p <0.01). *ns* = no significant differences.**
[1] Measures obtained in individual lobes.

with length ranging from 1,161.79 µm to 3,763.87 µm in accessions CMF-026 ('Solo') and CMF-247 ('Formosa'), respectively, and were covered with very small internal papillae and a large quantity of mucilage. The transmitting tissue was thin and centrally positioned throughout the length of the style, reaching the ovary (Figures 1g, 2k-l).

It is interesting to note the color variation of the stigmas in function of the floral development stage. In pre-anthesis, the stigmas had light green color, while in post-anthesis, i.e., when the flowers were mature and in the process of dehiscence, the stigmas had a yellowish color.

Numerous idioblasts containing druses were observed in the parenchymatous tissue, mainly in the stigmatic lobes, in all the accessions studied (Figure 1l-m). These druses are small crystals, aggregated in relatively spherical groups (Metcalfe & Chalk 2004). The presence of druses in plant tissues is related to an adaptation against herbivory and to maintain the ionic balance and development of the pollen tubes in the transmitting tissue, since their growth requires intracellular calcium gradients (Messerli et al. 2000, Holdaway-Clarke et al. 2003). According to Raven & Smith (1976), calcium oxalate can be extremely toxic to the metabolism of plants, and the formation of calcium crystals from oxalate can serve as a way to eliminate it.

Stigma receptivity

The stigma receptivity of *C. papaya* was significantly affected (p<0.001) by the variables (accessions and floral development states) as well as their interaction (Table III) according to the two methods evaluated (α-naphthyl acetate in combination with fast blue B salt, and hydrogen peroxide). In general, the greatest stigmatic receptivity was observed in anthesis and extended to post-anthesis (24 h after floral opening) (Table III).

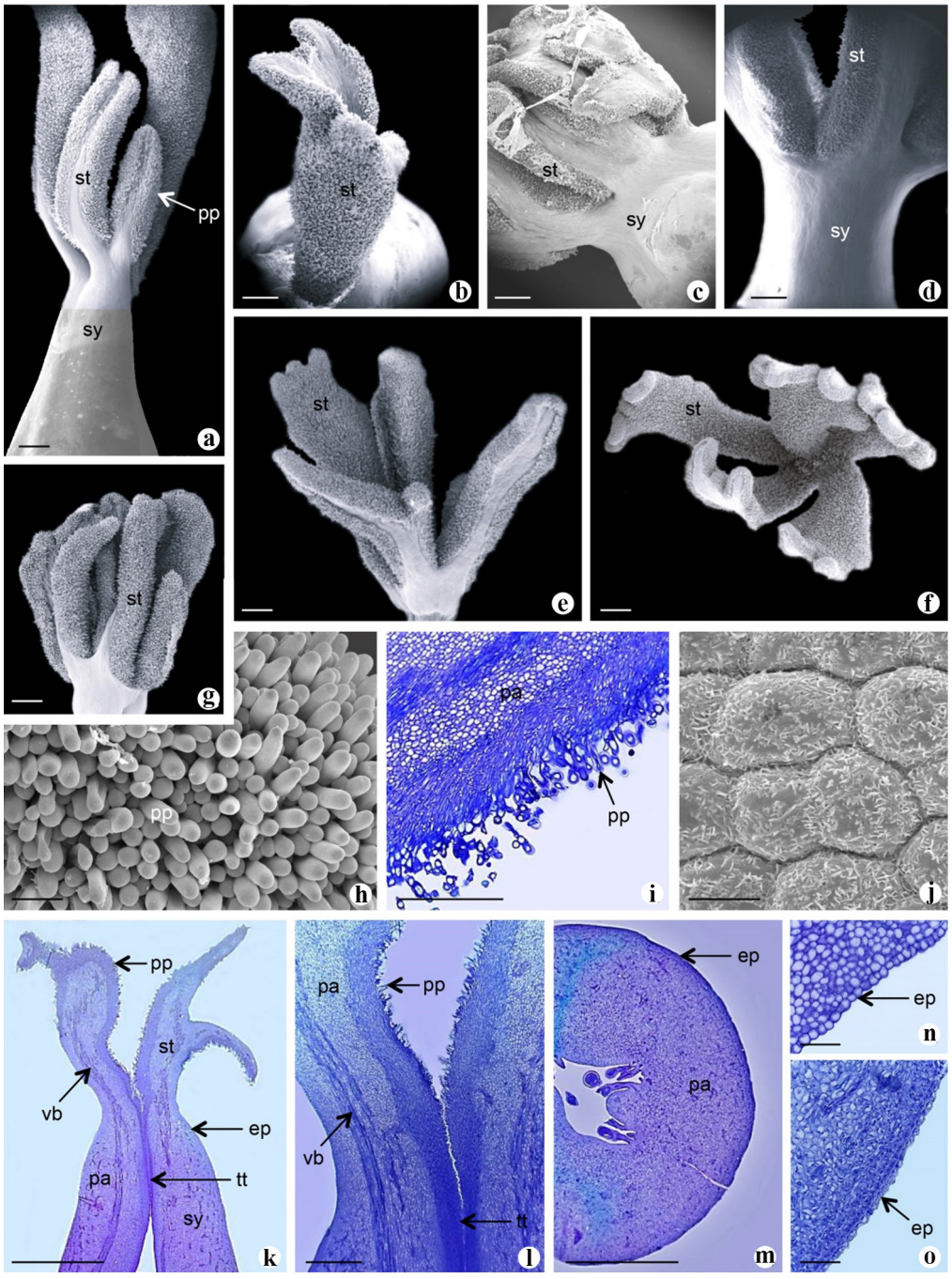

Figure 2. Morphoanatomy of the stigma/style in different papaya accessions (*C. papaya* L.) of the "Formosa' group. a) CMF-022; b) CMF-055; c) CMF-075; d) CMF-142; e-f) CMF-245; g) CMF-247; h) CMF-245; i) CMF-075; j) CMF-075; k-l) CMF-055; m) CMF-247; n) CMF-142; o) CMF-247. a-g, h, j) Morphological traits observed by scanning electron microscopy (SEM). i, k-o) Anatomic sections observed by light microscopy (LM). h) Adaxial surface of the stigmatic lobes showing the stigmatic papillae. i) Anatomic section showing the stigmatic papillae. j) Abaxial surface of the stigma showing the ornamentation of the stigmatic lobes. k-l) Longitudinal anatomic sections showing the transmitting tissue and stigmatic papillae. m) Transversal anatomic section of the base of the style. n) Anatomic section showing the epidermis of the style. o) Anatomic section showing the abaxial epidermis of the stigmatic lobes. ep = epidermis, pa = parenchyma, sp = stigmatic papillae st = stigma, sty = style, tt = transmitting tissue, vb = vascular bundle, dr = druses. Bars: a-g) = 500 µm; h-i, n-o) = 50 µm; k-m) = 200 µm.

Table III. Stigma receptivity of 10 *Carica papaya* accessions using α-naphthyl acetate + fast blue B salt and hydrogen peroxide at different stages of floral development.

Accession	Pre-anthesis	Anthesis	Post-anthesis
	α-naphthyl acetate + fast blue B salt		
CMF 020	(+) 1.00 aB	(++) 2.33 aA	(+++) 2.66 aA
CMF 022	(+) 1.00 aB	(++) 2.33 aA	(++) 1.66 bAB
CMF 026	(-) 0.33 bB	(+) 1.33 aA	(+++) 2.66 aA
CMF 055	(-) 0.00 bC	(+) 1.33 aB	(+++) 2.66 aA
CMF 070	(+) 0.66 aA	(+) 0.66 bA	(++) 1.66 bA
CMF 075	(-) 0.33 bB	(++) 2.33 aA	(+++) 2.66 aA
CMF 123	(-) 0.33 bB	(++) 2.00 aA	(++) 2.33 aA
CMF 142	(+) 0.66 aB	(+) 0.66 bB	(+++) 2.66 aA
CMF 245	(-) 0.33 bB	(+) 0.66 bB	(+++) 2.6 aA
CMF 247	(+) 1.33 aA	(++) 2.00 aA	(++) 1.66 bA
hydrogen peroxide			
CMF 020	(+) 1.33 aB	(++) 2.33 aB	(+++) 2.66 aA
CMF 022	(+) 0.66 aB	(+++) 3.00 aA	(+++) 3.00 aA
CMF 026	(-) 0.00 bB	(+++) 3.00 aA	(+++) 2.66 aA
CMF 055	(+) 0.66 aB	(++) 2.33 aA	(+++) 3.00 aA
CMF 070	(-) 0.33 bB	(++) 2.33 aA	(++) 2.33 aA
CMF 075	(-) 0.33 bB	(+++) 3.00 aA	(+++) 2.66 aA
CMF 123	(-) 0.00 bB	(++) 2.33 aA	(++) 2.00 aA
CMF 142	(-) 0.33 bB	(+++) 2.66 aA	(+++) 3.00 aA
CMF 245	(+) 0.66 aB	(+++) 3.00 aA	(+++) 2.66 aA
CMF 247	(+) 0.66 aB	(+++) 2.66 aA	(++) 1.66 bB

(-) no reaction; (+) weak positive reaction; (++) strong positive reaction; (+++) very strong positive reaction. Values correspond to the mean of the scores obtained from the repetitions. Means followed by the same lowercase letter in the column and uppercase letter in the row do not differ by the Scott-Knott test ($p<0.01$).

With the use of α-naphthyl acetate + fast blue B salt, the start of stigma receptivity was observed during pre-anthesis in the accessions CMF-020, CMF-22, CMF-70, CMF-142 and CMF-247 and in anthesis in the accessions CMF-026, CMF-055, CMF-075, CMF-123 and CMF-245, with weak positive reaction (+) and intensifying in the other floral development stages. The receptivity was more intense in post-anthesis, with a very strong positive reaction (+++) in 60% of the accessions (CMF-020, CMF-026, CMF-055, CMF-075, CMF-142 and CMF-245), while in this stage accessions CMF-22, CMF-070, CMF-123 and CMF-247 presented a strong positive reaction (++) (Table III, Figure 3).

The receptivity results when using hydrogen peroxide were similar to those with application of α-naphthyl acetate + fast blue B salt. The stigma receptivity started during pre-anthesis in accessions CMF-020, CMF-022 CMF-055, CMF-245 and CMF-247, with weak positive reaction (+). The strongest levels occurred in anthesis, with very strong positive reaction (+++) in accessions CMF-022, CMF-026, CMF-075, CMF-142, CMF-245

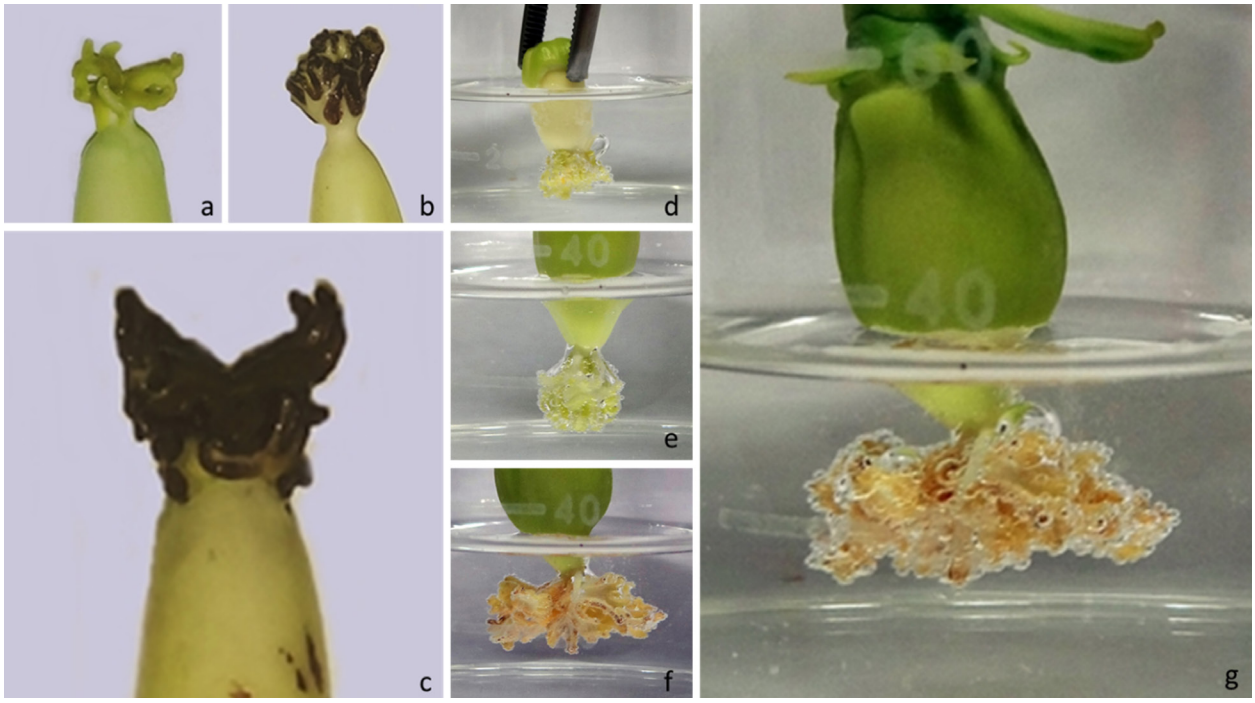

Figure 3. Evaluation of the stigmatic receptivity of papaya (*Carica papaya* L.) with α-naphthyl acetate + fast blue B salt (a-c) and hydrogen peroxide (d-g); a) No reaction in CMF-055 in pre-anthesis; b) Strong positive reaction (++) in CMF-020 in anthesis; c) Very strong positive reaction (+++) in CMF-026 in post-anthesis; d) Weak positive reaction (+) in CMF-245 in pre-anthesis; e) Strong positive reaction in CMF-070 in anthesis; f) Very strong positive reaction in CMF-026 in anthesis; g) Very strong positive reaction in CMF-142 in post-anthesis.

and CMF-247 and strong positive reaction (++) in accessions CMF-020, CMF-055, CMF-070 and CMF-123, increasing or remaining the same in post-anthesis, except for CMF-247, in which the receptivity declined. The lowest receptivity values were observed in accessions CMF-070 and CMF-123, irrespective of the method used and the floral stage (Table III, Figure 3).

Based on the two methods, it was possible to observe that the accessions had a particular pattern of reaction to each method. The stigmas subjected to α-naphthyl acetate presented a dark brown color when receptive, mainly in the region of the papillae. This can be related to the presence of exudates (enzymes), which are common in this region (Figure 3a-c). On the other hand, the treatment of the stigmas with hydrogen peroxide caused the appearance of small air bubbles (Figure 3d-g).

The results obtained demonstrate that the stigmas of some accessions were receptive before flower opening (pre-anthesis), with intensification of the enzyme reaction (esterase and peroxidase) until 24 hours after anthesis, a result also found by Parés et al. (2002) studying the 'Cartagena Amarilla' cultivar, and Rodrigues-Pastor et al. (1990), studying various cultivars of the two groups analyzed here ('Solo' and 'Formosa'). Analogously, Couto & Nacif (1999) observed that papaya stigmas were receptive from pre-anthesis until post-anthesis and maintained enzyme activity in post-anthesis in hermaphrodite and staminate flowers.

Parés et al. (2002) reported that in papaya plants, the stigmas can remain receptive for up to three days after anthesis, and the efficiency of artificial pollination was better in the first 48 hours after floral opening. Similar results were

observed by Damasceno Júnior et al. (2009), who reported greater stigma receptivity of papaya soon after anthesis, with receptivity continuing for up to 48 hours after floral opening.

Based on the characteristics of papaya plants and the data obtained in this study, we can suggest the hypothesis that since the floral buds have receptive stigmas in pre-anthesis and the pollen grains from hermaphrodite flowers are viable, the species presents cleistogamy, in which fecundation can occur even before floral opening. Cleistogamy was previously reported by Rodriguez-Pastor et al. (1990) for papaya plants of the 'Solo' group and by Damasceno Junior et al. (2009) for the cultivars 'Golden' ('Solo') and 'Tainung 01' ('Formosa'). Cleistogamy can pose a problem for genetic improvement programs, by making it impossible to guarantee the purity of the hybridizations. The emasculation of flowers is an alternative to overcome this phenomenon. This process consists of removing the anthers from the hermaphrodite flowers before they produce viable pollen and accomplish self-fecundation.

The results obtained in this study provide useful information for the planning and execution of programs for genetic improvement of papaya, by overcoming some incompatibility barriers to obtain lineages and hybrids, since the species can be self-fecundated without substantial loss of vigor (Dantas & Lima 2001). Therefore, the identification of the best floral development stage for stigmatic receptivity can maximize the chance of fertilization, and hence reduce the need for labor and time during the process. Additionally, and not less important, these results can support studies of the ecology and taxonomy of the species.

There is need to expand this study, by investigating other *C. papaya* accessions as well as other aspects related to reproductive biology, such as pollen germination *in vitro* and *in vivo*, controlled pollinations, formation of seeds and pollen-pistil interaction in the different floral development stages.

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